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14. ABSTRACT

In 2007, mutations in the SMARCB1 tumor suppressor (also known as INI1 and hSNF5), which lies in the familial schwannomatosis candidate region, were detected on a somatic and constitutional level in a single kindred with schwannomatosis. We have completed our study of 19 schwannomatosis kindreds who were unrelated to the best of the study participants' knowledge. Overall, we identified potentially causative constitutional alterations in 13 families, including 2 missense mutations (in 3 families), 4 splice site mutations, 2 additional mutations thought to be splice site mutations, and 4 changes of presumed pathogenicity in exon 9 (c.1240C>T). We compared haplotypes of the retained (affected) allele in tumorsfrom families with recurrent mutations in SMARCB1. Differing haplotypes were seen in 4 families with the c.1240C>T mutation in exon 9, which argues against a common ancestor. In contrast, a similar haplotype was seen in 2 families for c.158G>T mutation in exon 2 which suggests a common ancestor.

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Introduction

Neurofibromatosis (NF) encompasses a diverse group of genetic conditions whose common element is tumors of the nerve sheath. Schwannomatosis is a recently recognized third major type of NF, which results in multiple schwannomas without vestibular tumors diagnostic of NF2. Recent epidemiological studies have shown that schwannomatosis is as common as NF2. The objective of this project is to clone the locus responsible for familial schwannomatosis. We are exploring two competing hypotheses which address both the non random distribution of LOH observed in schwannomatosis tumors and the high rate of somatic *NF2* mutation seen along the *cis* allele. The first hypothesis is that schwannomatosis is due to mutation in a second tumor suppressor gene which lies near *NF2* on chromosome 22. In this model, schwannoma formation is dependent on four "hits" (two in the schwannomatosis tumor suppressor, and two in the linked *NF2* tumor suppressor). FISH results have suggested a second hypothesis in which a structural element facilitates loss of the *trans*-chromosome by increasing the rate of mitotic recombination. This is an especially attractive hypothesis since rates of mitotic recombination are both highly variable and genetically determined in humans.

Body

This section is organized around the approved statement of work.

Task 1. To develop a resource of study subjects and related biological materials (months 1 through 30): Task completed at time of last report.

Task 2. To refine the candidate region using LOH and linkage analysis (months 12 through 36): Task completed at the time of last report

Task 3. To determine the molecular mechanism leading to schwannomatosis (months 1 through 48): Subtasks a through d completed at time of last report.

e. Screening of candidate regions and/or loci based on results of task 2 (months 30 through 45).

In 2007, mutations in the *SMARCB1* tumor suppressor (also known as *INI1* and *hSNF5*), which lies in the familial schwannomatosis candidate region, were detected on a somatic and constitutional level in a single kindred with schwannomatosis (Hulsebos et al., 2007). The protein product of the *SMARCB1* locus is part of the human transcription complex and was cloned as the human homolog of yeast complex member *SNF5* in 1994 (Kalpana et al., 1994). Although it was originally identified as the binding partner for HIV-1 integrase (and was thus called *INI1),* in 1998 Versteege et al. mapped overlapping deletions in pediatric malignant rhabdoid tumors and found the smallest region of overlap was the *hSNF5* gene. In addition, point mutations were detected as the first hits in cell lines confirming that the molecule was a true tumor suppressor for this tumor type.

<u>Mutational analysis of familial schwannomatosis kindreds</u>

Since the last update, we have completed our study of 19 schwannomatosis kindreds who were unrelated to the best of the study participants' knowledge (Boyd et al, Clin Genet

2008). Genomic DNA from 6 non-founder EBV transformed cell lines, 12 schwannomas previously characterized at *NF2* and one meningioma in short term culture were used for the primary screen. The intron exon structure of the *SMARCB1* transcript was determined by comparison of the cDNA sequence (accession number AB017523) with the genomic sequence (AP000349 and AP000350). Primers were designed to amplify a minimum of 25 basepairs of flanking intronic sequence on either side of each exon. In addition, primers for exon 4 were designed to amplify the 51 basepairs included in the rare alternatively spliced form identified by two investigators (Mimori et al., 2002 and pers. com., Favre et al., 2003). Initial unidirectional automated sequencing of PCR products was performed on a single sample from each kindred; areas of poor sequencing, ambiguous or definite change were sequenced from the opposite direction or from primers internal to the amplification primers. Confirmed alterations found in tumor samples were immediately assessed in paired blood samples; alterations in blood samples were then assessed in affected and unaffected relatives. Because the *SMARCB1* locus is especially rich in polymorphism, all alterations were assessed in the single nucleotide polymorphism (SNP) database accessed through the NCBI website (http://www.ncbi.nlm.nih.gov/projects/SNP/). In addition to mutational analysis of the *SMARCB1* locus, we used microsatelite markers developed in previous years of funding to determine potential loss of heterozygosity of the region and potential identity by descent when identical changes were found in two or more families. A SALSA multiplex ligation-dependent probe amplification (MLPA) P258 SMARCB1 kit (MRC-Holland, Amsterdam, the Netherlands) containing 18 probes for the nine SMARCB1 exons and 23 control probes was used. Tumors entered in this screen had previously been analyzed at the *NF2* locus for both mutation and loss of heterozygosity as described in previous years' progress reports.

We detected no unreported alterations in exons 5, 7 and 8. A single change of unclear pathogenicity was seen in exon 9 (c.1240C.T) in specimens from families 1, 3, 5 and 10 but was not present in the panel of 50 unaffected individuals (see p. 9 for haplotype analysis).

Two missense alterations were detected in three kindreds but also absent from the unaffected panel. In exon 1, the change c.41C.A, p.Pro14- His was seen in a tumor from family 4. A second missense alteration was found in exon 2, c.158G.T, p.Arg53Leu. This change was seen in tumors from both family 9 and family PA-1 and the corresponding blood samples (see p. 9 for haplotype analysis).

Four mutations in conserved splice sites were detected in tumor material from four kindreds. In exon 3, c.36211G>A was found in a tumor from family E and the paired blood sample. In family PA-3, c.500G>A was seen at the ultimate base pair of exon 4 in a blood sample. In family 11, c.364G>T at the second base pair of exon 4 was present at a heterozygous level in a tumor specimen, the paired blood sample and two affected relatives and is presumed to affect the splice acceptor, although it could also result in p.Glu122X. Finally, c.79511G>T (exon 6) was detected in a blood sample from family 19 and an affected relative's blood sample.

Two additional potential splice site alterations were seen. In family V, a change that would produce a nonsense mutation in the rare long isoform of exon 4 was detected (c.50015G>T p.Gly16712X) with loss of the wild type G in a tumor specimen. The change was present at a heterozygous level in the paired blood sample and all affected relatives tested. In exon 6, a tumor from family P/Qu carried the change c.629-5T>G with partial loss of the wild type T. This alteration was present at a fully heterozygous level in paired normal tissue and in a blood specimen from an affected relative. Although these positions are not highly conserved in the mammalian splice acceptor, they may in fact influence splicing weakly.

MLPA analysis was performed on blood samples from non-founders in all 19 families. No decreased or increased copy number of any *SMARCB1* exon was seen.

A summary of mutational analysis combining the results described in this progress report with previous results obtained at the *NF2* locus in these samples is presented in table 1.

Table 1. Comparison of *NF2* mutational results to *SMARCB1* analysis

	SMARCB1 locus[a]				*NF2* locus[b]			
Family ID	Exon	Sequence	Effect[c]	LOH[d]	Exon	Sequence	Effect[c]	LOH[d]
4	1	c.41C>A	MS	No	10	c.979delG	FS	No
9	2	c.158G>T	MS	Yes	None	—	—	Yes
PA-1	2	c.158G>T	MS	Yes	8	c.778_782del	FS	Yes
E	3	c.362+1G>A	SP	Yes	7	c.600-1G>C	SP	Yes
11	4	c.364G>T	SP[e]	Yes	None	—	—	No
PA-3	4	c500G>A	SP	N/A	N/A			
V	4	c.500+5G>T	?SP	Yes	6	c.551G>A	NS	Yes
P/Qu	6	c.629-5T>G	?SP	No	None	—	—	Yes
19	6	c.795+1G>T	SP	N/A	N/A			
1	9	c.1240C>T	Unk	N/A	N/A			
3	9	c.1240C>T	Unk	Yes	3	c.241-3_260del	SP	Yes
5	9	c.1240C>T	Unk	Yes	7	c.630_631insT	FS	Yes
10	9	c.1240C>T	Unk	N/A	N/A			
8	None	—	—	N/A	N/A			
NH	None	—	—	Yes	2	c.179G>A	NS	Yes
Mo	None	—	—	No	None	—	—	No
PA-2	None	—	—	No	11	c1021C>T	NS	No
SD	None	—	—	N/A	N/A			
18	None	—	—	Yes	6	c.596delC	FS	Yes

Table 1. Comparison of previous *NF2* mutational results to *SMARCB1* analysis. All *NF2* gene mutations are somatic (in tumor tissue; not present in blood) and all *SMARCB1* gene alterations are constitutional. Predicted effect on the protein product is given as SP—splice site alteration, FS—frameshift, NS—nonsense mutation, MS—missense. LOH—loss of heterozygosity of surrounding microsatellite markers in tumor compared to blood. NA—no tumor available for analysis

Haplotype analysis of recurrent mutations

Overall, we identified potentially causative constitutional alterations in 13 families. Interestingly, six of these were accounted for by two recurrent alterations. In addition, we identified a third alteration that was previously reported in a separate study. Because incomplete penetrance may mask a relationship between these families, we sought to determine whether these recurrent alterations represented independent events or whether

the families harboring these mutations showed identity by descent as determined by haplotype analysis.

A missense alteration was found in exon 2, c.158G>T, in tumors and blood from both family 9 and family PA-1. We compared haplotypes of the retained (affected) allele in tumors from these two families using six microsatellite markers spanning approximately 1.2 Mbp around the SMARCB1 locus. An identical haplotype (135-160-225-341-201) was seen across five of six markers in these two families but not seen in control retained, affected alleles in tumor DNA from five other schwannomatosis families. According to the frequency of occurrence of each of these alleles in a panel of 50 unaffected, unrelated individuals (72%-25%- 5%-41%-80%), the probability of the affected alleles from these two families sharing this haplotype by chance is 0.003. There is no strong linkage disequilibrium between the two most informative markers, AB05 and AB09, which are the closest markers flanking the SMARCB1 locus. Thus, it is very likely that families 9 and PA-1 share a common ancestor. An unseen crossover event has presumably separated the D22S303 locus from the causative mutation in an intervening mitosis.

The point alteration, c.1240C>T, in exon 9 was found in four families (families 1, 3, 5 and 10) and was the most frequent alteration found in our cohort. This alteration lies in the 3'-untranslated region, 82 bp from the end of the native stop codon. Interestingly, each of these four families has a different haplotype, with at least three markers separating any two families. These results make descent from a common ancestor unlikely and suggest that this single base pair change may represent a recurrent causative mutation of SMARCB1.

<u>Unclear relationship between familial schwannomatosis and familial AT/RT syndrome</u>
A key issue is why the phenotype of these patients differs so drastically from those patients with rhabdoid predisposition syndrome who also bear constitutional mutations in the SMARCB1 transcript. At this time, it appears likely that both the location of mutation within the gene and the mutational type are critical to the final phenotype (Fig. 3).

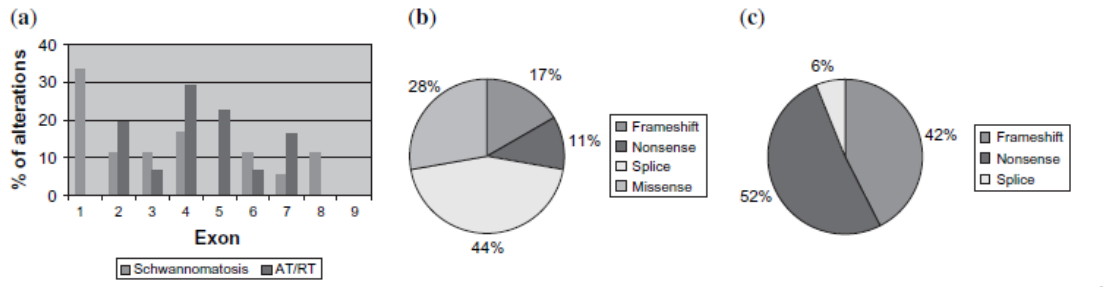

Fig. 3. Distribution and type of constitutional alterations[a] in *SMARCB1* in schwannomatosis patients compared with published reports from patients with rhabdoid predisposition syndrome. In (**a**), the relative frequency of constitutional alterations in exons 1–9 are listed for patients with schwannomatosis and rhabdoid predisposition syndrome. The relative frequency of alteration types are shown for schwannomatosis (**b**) and rhabdoid predisposition syndrome (**c**)[b]. [a]The predicted effect of sequence alterations were theoretically deduced rather than experimentally determined. [b]Some alterations that are predicted to cause nonsense mutations may cause splicing defects.

Key Research Accomplishments

- Development and validation of screening protocol for small alterations and multi-exon deletions in the *SMARCB1*gene.
- Detection of polymorphisms useful for further studies of identify by descent and expression.
- Confirmation of the hypothesis that *SMARCB1* is a tumor suppressor for schwannomas in the context of familial schwannomatosis.
- Reporting of the spectrum of *SMARCB1* mutations in a sample of familial schwannomatosis kindreds
- Preliminary identification of fundamental differences between *SMARCB1* mutations causing the phenotype of AT/RT and those causing schwannomatosis.

Reportable Outcomes

Presentations:
Invited presentations by the PI concerning this research were made to the Department of Biochemistry and Biophysics symposium, Oregon State University, Corvallis, OR 11/06, to the Washington State Neurofibromatosis Foundation 12/06 and to the Neurofibromatosis Mini Symposium, Heerlen, The Netherlands, 4/07.

An invited presentation by Ms. Chelsea Boyd concerning this research was made to the Children's Tumor Foundation, Park City, Utah (6/07).

Funding applied for:
The PI supported the submission of a proposal titled "Molecular characterization of *SMARCB*1 in familial and sporadic schwannomas" (PI:Scott Plotkin) to the NIH in June, 2007.

The PI supported the submission of a proposal titled "Effect of schwannomatosis-associated *SMARCB1(INI1)* mutations on cellular function, " (PI:Scott Plotkin) to the Children's Tumor Foundation in October, 2007.

The PI supported the submission of a proposal titled "Neurotrophin levels in schwannomas and schwannomatosis patients: is there a relationship to chronic pain?" (PI: Scott Plotkin) to the Children's Tumor Foundation in October, 2011. The proposal was funded.

Research opportunities:
The PI sponsored Dr. Miriam Smith as a postdoctoral fellow in the Department of Neurology at Massachusetts General Hospital, 5/07 to 5/2009.

Publications:
Boyd C, Smith MJ, Kluwe L, Balogh A, MacCollin M, and Plotkin SR. Alterations in the SMARCB1 (INI1) tumor suppressor gene in familial schwannomatosis. Clin Genet. 2008;74(4):358-366.

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Conclusions

Schwannomatosis is a third major form of NF, which epidemiological studies have shown is about as common as NF2. However, clinical recognition and molecular characterization have lagged far behind other forms of NF. The clarification of molecular alterations in schwannomatosis will likely have broad implications for other tumor suppressor gene syndromes. We have identified alterations in the *SMARCB1* gene which confirm that it is a tumor suppressor for familial schwannomatosis. This work has been replicated by other groups in England and Italy. Further work is needed to determine its role in other multiple and single tumor syndromes as well as the fundamental mechanisms that distinguish familial schwannomatosis from familial AT/RT syndrome.

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Appendices

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Original Article

Alterations in the *SMARCB1* (*INI1*) tumor suppressor gene in familial schwannomatosis

Boyd C, Smith MJ, Kluwe L, Balogh A, MacCollin M, Plotkin SR. Alterations in the *SMARCB1* (*INI1*) tumor suppressor gene in familial schwannomatosis.
Clin Genet 2008: 74: 358–366. © Blackwell Munksgaard, 2008

Schwannomatosis is a third major form of neurofibromatosis that has recently been linked to mutations in the *SMARCB1* (*hSnf5/INI1*) tumor suppressor gene. We analyzed the coding region of *SMARCB1* by direct sequencing and multiplex ligation-dependent probe amplification (MLPA) in genomic DNA from 19 schwannomatosis kindreds. Microsatellite markers in the *SMARCB1* region were developed to determine loss of heterozygosity (LOH) in associated tumors. We detected four alterations in conserved splice acceptor or donor sequences of exons 3, 4 and 6. Two alterations that likely affect splicing were seen in introns 4 and 5. An additional four alterations of unclear pathogenicity were found to segregate on the affected allele in eight families including two non-conservative missense alterations in three families. No constitutional deletions or duplications were detected by MLPA. Nine of 13 tumors examined showed partial LOH of the *SMARCB1* region consistent with 'second hits.' Alterations were detected in tumors both with and without somatic *NF2* gene changes. These findings support the hypothesis that *SMARCB1* is a tumor suppressor for schwannomas in the context of familial disease. Further work is needed to determine its role in other multiple and single tumor syndromes.

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Key words: INI1 – neurofibromatosis – schwannoma – schwannomatosis – SMARCB1 – tumor suppressor gene

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The neurofibromatoses are a diverse group of conditions that share a propensity to the development of multiple nerve sheath tumors. In both schwannomatosis (MIM 162091) and neurofibromatosis 2 [NF2 (MIM 101000)], those tumors are schwannomas, benign tumors resulting from expansion of the Schwann cell lineage. Schwannomatosis is differentiated from NF2 by the lack of involvement of the eighth cranial nerve (1). In addition, it has been observed that schwannomatosis patients do not develop other manifestations of NF2 such as ocular changes and ependymomas; a potential shared predisposition to meningioma has been suggested (1). Unlike NF1 and NF2, schwannomatosis is only rarely familial (approximately 15% of patients in our experience), and several kindreds have been described with incomplete penetrance (2).

Cloning of the constitutional alteration causing schwannomatosis has been hampered by the rarity of familial cases and the difficulty in separating schwannomatosis kindreds from mildly affected NF2 kindreds. Work from our own laboratory in a select group of well-defined families has placed the schwannomatosis locus on chromosome 22 in a 5-Mb candidate region situated approximately 3 Mb centromeric to the *NF2* gene itself [(2) and unpublished data]. In our experience, rigorously defined familial schwannomatosis patients do not carry constitutional *NF2* gene changes consistent with these linkage results. However, we and others have found a remarkable propensity for schwannomatosis tumors to bear inactivating, somatically acquired *NF2* gene mutations and loss of heterozygosity (LOH) of markers on the long arm of chromosome 22 (3, 4). This finding of restricted somatic mutation within a single tumor suppressor has thus far been unexplained and remains unprecedented in human tumor suppressor syndromes to our knowledge.

The protein product of the *SMARCB1* locus (also known as *hSNF5*, *INI1* and *BAF47*) is part of the human switch/sucrose non-fermentable chromatin-remodeling complex and was cloned as the human homolog of yeast complex member *SNF5* in 1994 (5). Subsequently, it was found to act as a true tumor suppressor in rhabdoid tumors of infancy and young childhood, including those with constitutional mutations and the rhabdoid predisposition syndrome (6, 7). A multitude of other tumor types have been examined for mutations in *SMARCB1* with variable results, including two studies of presumably sporadic schwannomas that failed to detect alterations (8, 9). *SMARCB1* lies within the familial schwannomatosis candidate region, and constitutional alterations with paired somatic mutation or LOH have been described in six schwannomatosis kindreds and several sporadically affected individuals (10–12). In this report, we determined the extent of both somatic and constitutional alterations of *SMARCB1* in a cohort of 19 schwannomatosis kindreds and their associated tumors.

Materials and methods

Subjects and specimen collection

We studied 19 schwannomatosis kindreds who were unrelated to the best of the participants' knowledge (Fig. 1). In all cases, the proband met criteria for 'definite' or 'presumptive' schwannomatosis (1). First-degree relatives and second-degree relatives were considered affected if they had one or more tumors radiographically consistent with schwannoma and/or one or more previously resected tumors with a pathology report of schwannoma. Persons were considered nonfounders if a parent was known to be affected or was an obligate carrier due to the affected status of other relatives. A panel of 50 unaffected and unrelated individuals was recruited from unaffected parents of sporadic NF1 and NF2 patients and persons married to schwannomatosis kindreds.

Blood samples were obtained from affected and unaffected individuals, and lymphoblast lines were established as described elsewhere (13). Excess tumor tissue was collected at the time of diagnostic or therapeutic procedures after pathologic studies were complete or was collected at the time of autopsy. G401 tumor cells derived from a human rhabdoid tumor of the kidney (14) were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 50 U penicillin/50 μg streptomycin per milliliter. High-molecular-weight DNA was extracted from peripheral blood leukocytes, cultured lymphoblasts, frozen pulverized tumor, cultured tumor and normal tissues obtained at autopsy using a PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN). This study was approved by the Institutional Review Board of Partners HealthCare, and informed consent was obtained from all living individuals donating tissue to this work.

Direct sequencing of *SMARCB1*

The intron–exon structure of the *SMARCB1* transcript was determined by comparison of the complementary DNA sequence (accession number AB017523) with the genomic sequence (AP000349 and AP000350; Table S1, supplementary material online). Primers were designed to amplify a minimum of 25 base pairs (bp) of flanking intronic sequence on either side of each exon (Table S2, supplementary material online). In addition, primers for exon 4 were designed to amplify the 51 or 54 bp included in the rare alternatively spliced form identified by two investigators (15, 16). Initial unidirectional automated sequencing of polymerase chain reaction products was performed on a single sample from each kindred; areas of poor sequencing, ambiguous or definite change were sequenced from the opposite direction or from primers internal to the amplification primers. Confirmed alterations found in tumor samples were immediately assessed in paired blood samples; alterations in blood samples were then assessed in affected and unaffected relatives. Because the *SMARCB1* locus is especially rich in polymorphism, all alterations were assessed in the single nucleotide polymorphism (SNP) database accessed through the National Center for Biotechnology Information Web site. Non-truncating alterations of unclear pathogenicity were also assessed in a panel of 50 unaffected unrelated individuals.

Multiplex ligation-dependent probe amplification analysis of *SMARCB1*

DNA concentration of genomic samples was measured using a Nanodrop ND-1000 spectrophotometer. Twenty to 500 ng were used for each assay. A SALSA multiplex ligation-dependent probe amplification (MLPA) P258 *SMARCB1* kit (MRC-Holland, Amsterdam, the Netherlands) containing 18 probes for the nine *SMARCB1* exons and 23 control probes was used. Hybridization,

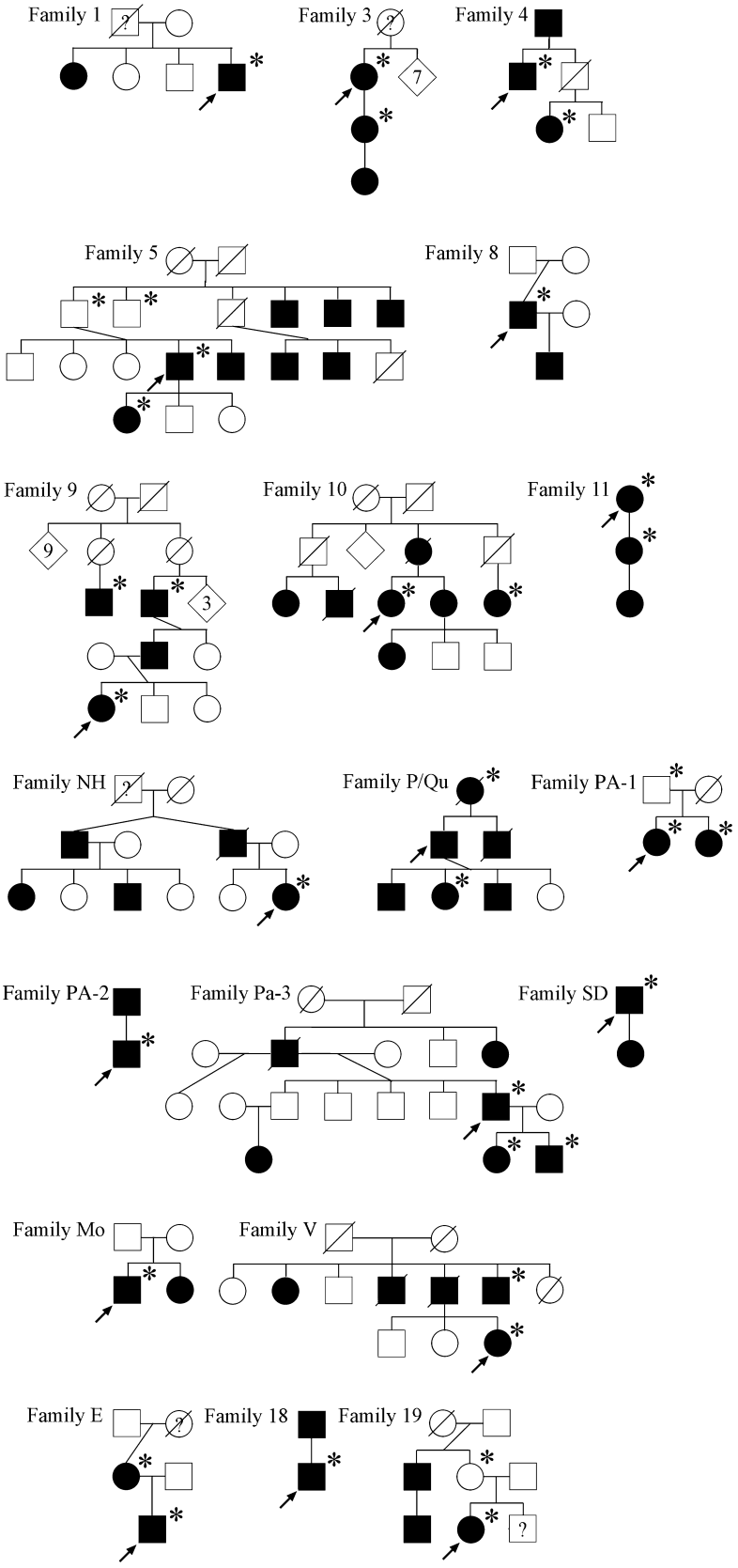

Fig. 1. Families studied in this report. Families 1, 3, 4, 5, 8, 9, 10 and 11 have been previously reported (1, 13). The remaining families have not previously been reported to the best of our knowledge. →, proband; *, affected family member used to assess *SMARCB1* alteration; ?— felt to be affected by family members but no pathology reports available.

ligation and amplification of the probes were carried out as per the manufacturer's instructions. Ten microliters of the amplification product were analyzed using an ABI 3730 DNA Analyzer, with Biomek FX robotics and with GeneScan 500 LIZ (Applied Biosystems, Foster City, CA) as the internal size standard. Data analysis was performed by exporting the peak area values to an Excel file and calculating the relative probe signals by dividing each measured peak by the sum of all peak areas for that sample. Ratios of relative peak signals for each test sample were calculated by dividing the relative peaks of each test sample by the average peak signals of four control samples derived from unaffected individuals.

Microsatellite markers in the *SMARCB1* region

Primers were designed to amplify regions surrounding dinucleotide, trinucleotide and tetranucleotide repeats detected by the Genome Browser Gateway of the University of California, Santa Cruz Genome Bioinformatics service (http://genome.ucsc.edu; Table S3, supplementary material online). In selected cases, the centromeric marker D22S303 (21,604 kbp in build 36.2) and the telomeric marker D22S1174 (22,818 kbp) were amplified using primers and protocols available at the GDB Human Genome Database Web site (http://gdbwww.gdb.org/). Primers were labeled with fluorescence dyes, and amplification products were separated on an automated Genetic Analyzer ABI 3730XL or ABI 310. LOH in tumor specimens compared with blood samples was determined by visual inspection conducted independently by at least two readers. When ambiguous results were obtained, additional markers were compared until consensus was reached.

NF2 locus analysis

Analysis of the *NF2* locus was performed as previously described (17). Briefly, the 15 exons of the *NF2* gene known to harbor pathogenic mutations were amplified from genomic tumor DNA and directly sequenced or scanned using single-strand conformational polymorphism or temperature-gradient gel electrophoresis. When aberrations were detected with the later methods, direct sequencing was subsequently performed. All confirmed changes were sequenced bidirectionally. Loss of the *NF2* region on chromosome 22 was determined using a panel of flanking and intragenic microsatellite markers spanning 5 Mb as previously described (17).

Results

Sequence alterations in *SMARCB1*

Initial sequencing was performed on genomic DNA from 6 non-founder lymphoblastoid cell lines, 12 flash-frozen schwannomas and 1 meningioma in short-term culture. No unreported alterations were detected in exons 5, 7 and 8. Informativeness at known SNPs was seen in a small number of samples in exons 5 (accession number rs5751738), 6 (rs2070457), 7 (rs35817983 and rs2229354 that appear to be in linkage disequilibrium), and 9 (rs34399789). In exon 5, rs5760030 was observed to be GG or G/- in all samples with a complete absence of the A allele. Two constitutional changes that are unreported in the SNP database but are unlikely to be pathogenic were seen in exon 1 [c.93+105GC(7_9) and c.93+121_93+122dup(TC)] in several samples. In exon 3, c.233-43T>A was present in a tumor specimen from family 11 and the paired blood sample; however, the affected parent and child did not carry the change, indicating that it was on an unaffected allele.

A single change of unclear pathogenicity was seen in exon 9 (c.1240C>T) in specimens from families 1, 3, 5 and 10 but was not present in the panel of 50 unaffected individuals. This change lies 82 bp from the end of the native stop codon in the 3′ untranslated region. Tumor specimens from families 3 and 5 showed partial or complete loss of the wild type C in comparison to the T on chromatograms. It remains unclear if this is due to a contamination by normal tissue in the tumor specimen or a portion of schwannoma cells that are not deleted in this area. Affected relatives in families 3, 5, and 10 were heterozygous for the change.

Two missense alterations were detected in three kindreds but also absent from the unaffected panel. In exon 1, the change c.41C>A, p.Pro14-His was seen in a tumor from family 4. There was no loss of the wild type C in this tumor, and the alteration was present in the paired blood sample and in other affected family members. This is a non-conservative amino acid change, which is identical to the level of *Drosophila* (accession AAF52024). A second missense alteration was found in exon 2, c.158G>T, p.Arg53Leu. This change was seen in tumors from both family 9 and family PA-1 and the corresponding blood samples. Both tumors showed loss of the wild type G when compared with the fully heterozygous paired blood samples. The change was present in all affected family members examined from both families. This is also a non-conservative amino acid change, which is identical to the level of *Xenopus* (accession AAH767140).

Four mutations in conserved splice sites were detected in tumor material from four kindreds. In exon 3, c.362+1G>A was found in a tumor from family E and the paired blood sample. There was no clear LOH in the sequencing of tumor compared with blood (although LOH was seen at flanking microsatellite markers), and the alteration was present in an affected family member. In family PA-3, c.500G>A was seen at the ultimate base pair of exon 4 in a blood sample. This change was present in all affected family members sequenced. This alteration of a highly conserved splice donor position could have multiple effects. If it encouraged a greater expression of the alternatively spliced long form of exon 4 (15, 16), an in-frame stop codon would be produced (p.Trp167X). If splicing were maintained to exon 5, a missense change would be produced (p.Cys167Tyr). Similarly, in family 11, c.364G>T at the second base pair of exon 4 was present at a heterozygous level in a tumor specimen, the paired blood sample and two affected relatives and is presumed to affect the splice acceptor, although it could also result in p.Glu122X. Finally, c.795+1G>T (exon 6) was detected in a blood sample from family 19 and an affected relative's blood sample.

Two additional potential splice site alterations were seen. In family V, a change that would produce a nonsense mutation in the rare long isoform of exon 4 was detected (c.500+5G>T p.Gly167+2X) with loss of the wild type G in a tumor specimen. The change was present at a heterozygous level in the paired blood sample and all affected relatives tested. In exon 6, a tumor from family P/Qu carried the change c.629-5T>G with partial loss of the wild type T. This alteration was present at a fully heterozygous level in paired normal tissue and in a blood specimen from an affected relative. Although these positions are not highly conserved in the mammalian splice acceptor, they may in fact influence splicing weakly (18).

Copy number analysis of *SMARCB1*

MLPA analysis was performed on blood samples from non-founders in all 19 families. No decreased or increased copy number of any *SMARCB1* exon was seen. G401 tumor cells showed decreased copy number in all 18 probes located within *SMARCB1* exons but none of the 23 control probes (data not shown). LOH at two or more markers in the *SMARCB1* region was seen in 8 of 12 schwannomas and a meningioma from family 9. In most cases, loss was incomplete, suggesting that a significant population of cells within the tumor retains the wild-type allele (Fig. 2).

Mutational analysis of the *NF2* locus

Mutational analysis of the coding regions of the *NF2* gene in these 13 tumors revealed typical truncating mutations in 9 (69%; Table 1). Consistent with our previous observations (2), mutations were clustered in exons 1 through 8, which encodes the protein 4.1 domain of the NF2 protein product. Single mutations were detected in exons 10 and 11, and no mutations were detected in exons 12 through 15. Family E was found to carry a constitutional private polymorphism (c.1340+24C>T); all other changes were somatic. Microsatellite analysis revealed partial LOH of two or more markers in 8 of 12 schwannomas and the meningioma. In one tumor, loss was appreciated at the *SMARCB1* locus but not at the *NF2* locus, and in a second, loss was seen at *NF2* but not at *SMARCB1* locus. *NF2* mutations were detected in both tumors with and without *SMARCB1* alterations (Table 1).

Discussion

Our study of schwannomatosis kindreds reveals that over two-thirds (13 of 19, 68%) segregate constitutional alterations in the *SMARCB1* transcript. Although a portion of these are of unclear pathogenicity, the four changes in highly conserved splice sites almost certainly cause significant changes in the protein product of this transcript. LOH was frequent but not universal, and the possibility remains that other mechanisms of mutations such as intronic changes causing splicing alterations or promoter mutations account for some constitutional and somatic events not detected.

The single change in exon 9 (c.1240C>T) in four unrelated families is the most frequent mutation found in our cohort. This point mutation lies 82 bp from the end of the native stop codon in the 3′ untranslated region; interestingly, should the native stop be read through, an in-frame nonsense change would be produced (CGA>TGA); however, the significance of this change is not currently apparent. Although the pathological consequence of this change is yet unknown, lack of this alteration in a panel of 50 unaffected individuals suggests its association with schwannomatosis. Furthermore, immunohistochemical analysis of SMARCB1 protein in schwannomas from families 1, 3, and 10 revealed a mosaic pattern

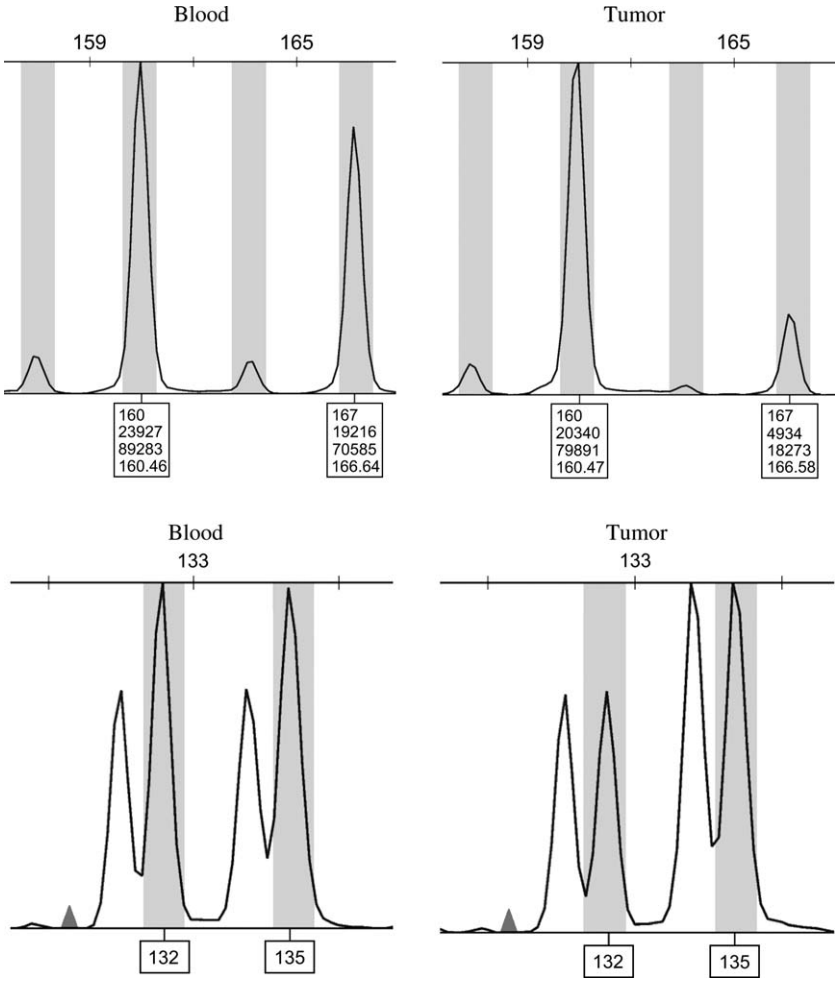

Fig. 2. Microsatellite analysis of blood tumor pairs. In the top panel, nearly complete loss of the 167 allele of marker AB05 is seen in this meningioma from family 9 compared with the blood sample. In the bottom panel, an incomplete loss of the 132 allele of marker AB02 is seen in a schwannoma from family E compared with the blood sample.

consistent with loss of protein expression in a subset of tumor cells (19). It would be interesting to examine this effect of this alteration in an *in vitro* cell culture model.

Two recurrent alterations in six families were identified in this study: c.1240C>T in families 1, 3, 5 and 10 and c.158G>T in families 9 and PA-1. In addition, the c.41C>A alteration in family 4 has been reported previously (12). The increased frequency of alterations at these base pairs can be explained either by descent from a common ancestor or by increased susceptibility to mutation (i.e. hotspots). In this study, none of the families is known to be related. However, descent from a common ancestor cannot be ruled out because none of these families can identify a founder and they live in geographic proximity. Recurrent constitutional mutations will require an analysis of haplotype before concluding that they are independent occurrences.

Several clinical caveats should be noted with regards to these data. We studied only patients with a family history of the disease. Constitutional alterations in the *SMARCB1* locus are less common in patients with sporadic schwannomatosis, which underscores the genetic heterogeneity of this condition (11, 12, 20). We also limited our initial screening to tumors or blood specimens from non-founders because somatic mosaicism has been a limiting factor in the detection of mutations in other forms of NF (21). Our kindreds were rigidly defined as not having vestibular tumors, with a directed skull base magnetic resonance imaging (MRI) performed to specific protocol in adulthood. Small vestibular tumors may in fact be missed on standard MRI studies (1), leading to the misclassification of kindreds with milder forms of NF2 as being schwannomatosis families. Because these kindreds will bear constitutional mutations in the *NF2* and not the *SMARCB1* transcript, their inclusion in studies of the latter will lead to falsely low detection rates.

Including the alterations described in this report, a total of 18 coding and/or splice site alterations have now been detected on a constitutional level in schwannomatosis patients including 8 splice site mutations, 2 nonsense mutations, 3 frameshifting mutations and 5 missense alterations

Table 1. Comparison of *NF2* mutational results to *SMARCB1* analysis

	SMARCB1 locus[a]				*NF2* locus[b]			
Family ID	Exon	Sequence	Effect[c]	LOH[d]	Exon	Sequence	Effect[c]	LOH[d]
4	1	c.41C>A	MS	No	10	c.979delG	FS	No
9	2	c.158G>T	MS	Yes	None	—	—	Yes
PA-1	2	c.158G>T	MS	Yes	8	c.778_782del	FS	Yes
E	3	c.362+1G>A	SP	Yes	7	c.600-1G>C	SP	Yes
11	4	c.364G>T	SP[e]	Yes	None	—	—	No
PA-3	4	c500G>A	SP	N/A	N/A			
V	4	c.500+5G>T	?SP	Yes	6	c.551G>A	NS	Yes
P/Qu	6	c.629-5T>G	?SP	No	None	—	—	Yes
19	6	c.795+1G>T	SP	N/A	N/A			
1	9	c.1240C>T	Unk	N/A	N/A			
3	9	c.1240C>T	Unk	Yes	3	c.241-3_260del	SP	Yes
5	9	c.1240C>T	Unk	Yes	7	c.630_631insT	FS	Yes
10	9	c.1240C>T	Unk	N/A	N/A			
8	None	—	—	N/A	N/A			
NH	None	—	—	Yes	2	c.179G>A	NS	Yes
Mo	None	—	—	No	None	—	—	No
PA-2	None	—	—	No	11	c1021C>T	NS	No
SD	None	—	—	N/A	N/A			
18	None	—	—	Yes	6	c.596delC	FS	Yes

[a]All *SMARCB1* gene alterations are constitutional and were confirmed in blood samples or lymphoblastoid lines.
[b]*NF2* gene alterations are somatic and were identified in tumor specimens only.
[c]Predicted effect on the protein product is given as SP, splice site alteration; FS, frameshift; NS, nonsense mutation; MS, missense; Unk, unknown; N/A, no tumor available for analysis.
[d]Loss of heterozygosity (LOH) is that seen at microsatellite markers and at times differed from subtle LOH of sequence seen on chromatograms.
[e]Presumed to affect splice acceptor but also could result in nonsense alteration.

(10–12). A key issue is why the phenotype of these patients differs so drastically from those patients with rhabdoid predisposition syndrome who also bear constitutional mutations in this transcript. At least 21 unrelated individuals and 6 unrelated kindreds with rhabdoid tumors have been reported in the literature with constitutional mutations in the *SMARCB1* gene [(6, 7, 22–33); Fig. 3]. Three families include adults who are constitutional carriers of mutation yet have not developed any known tumors, suggesting that there is a 'window' of childhood opportunity of the development of rhabdoid tumors for those bearing *SMARCB1* mutation (28, 30, 34). However, this alone cannot explain the dichotomy between schwannomatosis and rhabdoid disposition populations because to date, there has been no published report of an adult rhabdoid tumor survivor or carrier developing schwannoma(s) or of a schwannomatosis kindred including a childhood rhabdoid tumor patient. Instead, it appears likely that both the location of mutation within the gene and the mutational type are critical to the final phenotype. For example, constitutional rhabdoid tumor-associated *SMARCB1* mutations have not been reported within exon 1 (where 6 of 18 schwannomatosis mutations are located). Furthermore, while 96% (26 of 27) of constitutional mutations in rhabdoid tumor patients are truncating mutations, that is nonsense or frameshift, less than 33% (5 of 18) of mutations in schwannomatosis patients are truncating, and 4 of these 5 lie in exon 1. Further work is needed to understand how the molecular biology of mutation within the *SMARCB1* transcript results in these widely variable outcomes.

Co-mutation of both the *NF2* and the *SMARCB1* was found in the majority of tumors studied in this report and was also seen in the reports of Sestini et al. (11) and Hadfield et al. (12). Because these genes lie in close proximity on the long arm of chromosome 22, a single LOH event may precipitate haploinsufficiency in both and accelerate tumor formation as has been observed in mouse models of NF1 and p53 co-mutation (35, 36). Similar findings of co-mutation in the *NF2* and *SMARCB1* transcripts have been reported in sporadic meningiomas. In one study of 126 meningiomas, somatically acquired c.1130G>A (p.Arg377His) was detected in 4, and 3 of 4 four tumors also bore typical truncating mutations of the *NF2* gene (37). In a second study of 80 meningiomas analyzed in four of the nine *SMARCB1* exons only, an exon 9 frameshifting mutation was detected in one (38). Similar to our observations and those of Hulsebos et al. (10), this mutation was seen in only a subpopulation of tumor cells. Further work is needed to understand the presumed mixture of null and

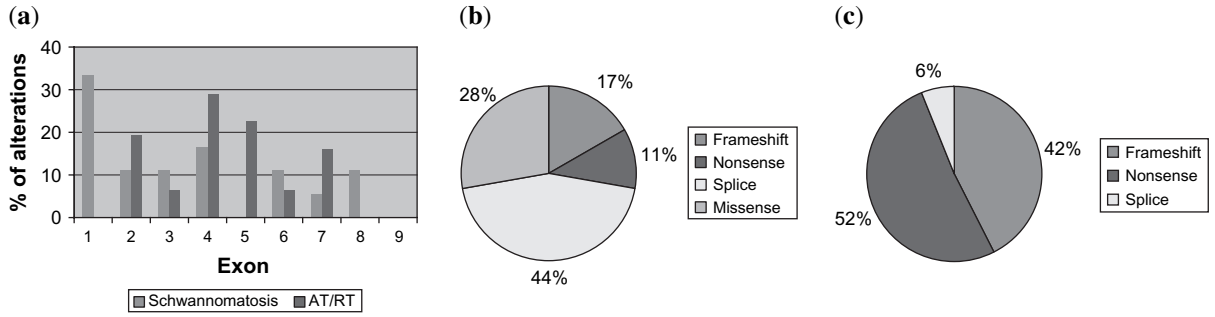

Fig. 3. Distribution and type of constitutional alterations[a] in *SMARCB1* in schwannomatosis patients compared with published reports from patients with rhabdoid predisposition syndrome. In (**a**), the relative frequency of constitutional alterations in exons 1–9 are listed for patients with schwannomatosis and rhabdoid predisposition syndrome. The relative frequency of alteration types are shown for schwannomatosis (**b**) and rhabdoid predisposition syndrome (**c**)[b]. [a]The predicted effect of sequence alterations were theoretically deduced rather than experimentally determined. [b]Some alterations that are predicted to cause nonsense mutations may cause splicing defects.

haploinsufficient cells in these tumors and how haploinsufficiency at one of these two loci may affect or accelerate mutation at the other.

In conclusion, our findings support the hypothesis that *SMARCB1* is a tumor suppressor for schwannomatosis in the context of familial disease, a hypothesis also supported by our parallel immunohistochemical studies (19). Co-mutation of the *NF2* gene and the *SMARCB1* gene implies that in at least some schwannomas, a sequence of events more complex than the classic 'two hit' model is required for tumorigenesis because some tumors contain *SMARCB1* mutations, *NF2* mutations and LOH. We are currently employing alternative methods in these families to detect mutations such as intronic changes or promoter alterations, which would elude the exon-based approach reported in this study. Additional work is needed to show how frequently *SMARCB1* functions as a tumor suppressor in the context of other multitumor syndromes such as sporadic schwannomatosis and NF2 and the more common phenomenon of single sporadic vestibular and non-vestibular schwannomas.

Supplementary material

Table S1. Intron–exon structure of the *SMARCB1* transcript.
Table S2. Primers used for amplification and sequencing of *SMARCB1*.
Table S3. Microsatellite markers developed in the *SMARCB1* region.
Supplementary materials are available as part of the online article at http://www.blackwell-synergy.com

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Letter to the Editor

Identity analysis of schwannomatosis kindreds with recurrent constitutional *SMARCB1 (INI1)* alterations

To the Editor:

Schwannomatosis is a form of neurofibromatosis that is characterized by the formation of multiple schwannomas without the vestibular involvement found in neurofibromatosis type 2. Familial schwannomatosis is characterized by widely variable expressivity and incomplete penetrance, making full ascertainment of families difficult (1). The *SMARCB1* tumor suppressor gene, which lies in the familial schwannomatosis candidate region, has recently been found to harbor alterations in both familial and sporadic schwannomatosis patients (2,3,4,5). As would be expected, tumor material from these patients frequently shows loss of heterozygosity of the unaffected allele and retention of the affected, transmitted and mutation-bearing allele. In our own study of 19 schwannomatosis kindreds, we identified potentially causative constitutional alterations in 13 families. Interestingly, six of these were accounted for by two recurrent alterations (5). In addition, we identified a third alteration that was previously reported in a separate study (4). Because incomplete penetrance may mask a relationship between these families, we sought to determine whether these recurrent alterations represented independent events or whether the families harboring these mutations showed identity by descent as determined by haplotype analysis.

Two families in our cohort (families 9 and PA-1) bore the same missense alteration (c.158G>T) in exon 2, which leads to a non-conservative amino acid change (p.Arg53Leu) at an evolutionarily conserved residue. The alteration was present in all affected family members examined from both families and was not found in a screen of 100 unaffected, unrelated alleles. Family 9 is a multigenerational family with three generations known to be affected with schwannomatosis. Family PA-1 has two generations with one generation known to be affected (5). Although these two families have no known relationship, neither family can identify a founder and they live in geographic proximity. We compared haplotypes of the retained (affected) allele in tumors from these two families using six microsatellite markers spanning approximately 1.2 Mbp around the *SMARCB1* locus (Table 1). Primers for the markers *D22S303* and *D22S1174* were found on the GDB Human Genome Database website (http://gdbwww.gdb.org/). The markers AB02, AB05, AB09 and AB03, have been described previously (5). An identical haplotype (135-160-225-341-201) was seen across five of six markers in these two families but not seen in control retained, affected alleles in tumor DNA from five other schwannomatosis families (Table 1). According to the frequency of occurrence of each of these alleles in a panel of 50 unaffected, unrelated individuals (72%-25%-5%-41%-80%), the probability of the affected alleles from these two families sharing this haplotype by chance is 0.003. There is no strong linkage disequilibrium between the two most informative markers, AB05 and AB09, which are the closest markers flanking the *SMARCB1* locus. Thus, it is very likely that families 9 and PA-1 share a common ancestor. An unseen crossover event has presumably separated the *D22S303* locus from the causative mutation in an intervening mitosis.

The point alteration, c.1240C>T, in exon 9 was found in four families (families 1, 3, 5 and 10) and was the most frequent alteration found in our cohort. This alteration lies in the 3′-untranslated region, 82 bp from the end of the native stop codon. Tumor specimens from families 3 and 5 showed partial or complete loss of the wild-type C in comparison to the altered T on chromatograms. The haplotypes of the affected alleles were determined from tumor material with loss of heterozygosity in families 3 and 5 and from somatic hybrid lymphoblast cell lines containing only the alteration-bearing chromosome 22 in families 1 and 10. Interestingly, each of these four families has

Table 1. Haplotype analysis across the *SMARCB1* region of affected alleles from schwannomatosis families containing recurrent constitutional alterations

	Centromeric			Telomeric		
Family[b]	*D22S303* 21,605[a]	AB02 22,294	AB05 22,367	AB09 22,769	*D22S1174* 22,819	AB03 22,840
PA-1[c]	223	135	160	225	196	201
9	225	135	160	225	196	201
1[d]	214	132	157	233	200	201
3	226	132	166	217	198	201
5	226	135	157	209	196	201
10	226	132	166	229	202	198
Control tumors[e]						
V	224	135	157	227	194	202
NH	223	135	160	215	194	201
18	223	135	157	221	196	202
E	213	135	160	225	202	201
11	223	132	157	211	192	202

[a]Genomic location of markers on chromosome 22 in kilobase pairs (NCBI build 36.3).
[b]Haplotype analysis is of the affected, transmitted allele as determined by allele retention in tumor material or through segregation in somatic hybrid cell lines.
[c]Families PA-1 and 9 share the alteration, c.158G>T.
[d]Families 1, 3, 5 and 10 share the alteration c.1240C>T.
[e]Controls are the retained, affected alleles from tumors of other schwannomatosis kindreds.

a different haplotype, with at least three markers separating any two families (Table 1). These results make descent from a common ancestor unlikely and suggest that this single base pair change may represent a recurrent causative mutation of *SMARCB1*.

In this study, we determined that two apparently unrelated families, sharing an exon 2 missense alteration, may in fact have a common founder. However, we found four families sharing a novel alteration in the 3′-untranslated region to be unrelated by haplotype analysis. A similar study of haplotype analysis, carried out for causative changes in melanoma (*CDKN2A*) families, has shown some recurrent alterations to be true independent mutational events and others to be due to a founder effect (6). Our results suggest that there may also be regions of the *SMARCB1* gene that are more prone to mutation than others but that families harboring recurrent constitutional alterations require an analysis of haplotype before concluding that they are independent occurrences.

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